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Role of Phe95 In the Receptor Binding of Influenza B Virus Hemagglutinin

by

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ABSTRACT

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Influenza A and B viruses are significant human pathogens responsible for the annual seasonal "flu". Diverged some 2000 years ago, influenza B virus has several important differences from influenza A virus, including lower receptor-binding affinity and very limited host range. Based on sequence comparison and our prior structural studies, we hypothesized that a key difference in the receptor-binding site of influenza virus hemagglutinin (HA), phenylalaline (Phe) 95 in influenza B virus HA (BHA), versus tyrosine (Tyr) in influenza A virus HA (AHA), is possibly the molecular basis for the different receptor-binding affinity. We further hypothesized that this could be at least partially responsible for the very limited host range of influenza B virus. By using glycan and red blood cell binding assays, we demonstrated that the mutation Phe95→Tyr in BHA substantially enhanced receptor-binding affinity. Furthermore, this mutation efficiently competed against the infection of influenza A virus and greatly improved the binding of BHA to three mammalian cell lines. Taken together, residue 95 of BHA appears to be a key determinant for the receptor binding affinity and host range of influenza B virus.

Abbreviations:

HA: Hemagglutinin; BHA: Influenza B virus Hemagglutinin; SA: Sialic Acid; RBC: Red Blood Cell; MDCK: Madin Darby Canine Kidney.

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Table of Contents

List of Tables

List of Figures

1. Introduction

Great efforts have been dedicated to the understandings of the replication of influenza viruses, where each step of the virus infection might be a potential target for prophylactic and therapeutic treatment. The discernable phases of influenza virus replication can be briefly described as: 1) Virus attachment and entry to the host cell; 2) Virion assembly; and 3) Release from the host cell (1). Profound progresses were made during the recent years from different scientific research fields. Structural characterizations of several important proteins of influenza viruses provide the templates for drug designs, including the envelop glycoproteins hemagglutinin (HA) and neuraminidase (NA), M2 ion-channel protein, the subunits (PA, PB1 and PB2) of viral polymerase complex and nonstructural (NS) protein (see reference 2 for some of the solved structures).

One of the unsolved issues is that the underlying mechanism that accounts for the different host ranges for influenza A and B viruses, which are two main types of influenza virus responsible for the seasonal "flu" epidemics each year. Based on the sequence comparison and our recent X-ray structure of the influenza B virus hemagglutinin (BHA), which mediates the attachment of the virus to the receptors on the host cell, show that its receptor binding site has a phenylalaline (Phe) at position 95, versus tyrosine (Tyr) in influenza A virus HA (3). We hypothesize that this key difference is possibly the molecular basis for the different receptor-binding affinity and this could be at least partially responsible for the very limited host range of influenza B virus.

In order to test this hypothesis, we make the mutation Phe95→Tyr on BHA for biochemical and biological studies. Although naturally occurring influenza B viruses frequently have asparagine (Asn) and serine (Ser) at HA1 194 and 196 respectively, making a glycosylation site at Asn194, there are cases where this glycosylation site was abolished in, for example, some field isolates (4, 5), and egg-adapted variants (6-12), as a result of mutations at HA1 194 and 196. Thus, to comprehensively study the effects of residue at position 95, Phe versus Tyr, we constructed the mutation Phe95→Tyr on both background sequences, with a glycosylation site at Asn194 (wild type), or without a glycosylation site (Asp194, Asn194→Asp).

2. Methods and materials

2.1. Cloning, expression and purification of BHA and its mutants

The DNA of hemagglutinin of influenza B/YM/73 (BHA) virus was obtained by RT-PCR. The BHA fragment was cloned to pRB21 plasmid to facilitate the expression in vaccinia systems. The site-directed mutagenesis was used to make the Phe95→Tyr, Asn194→Asp and Phe95→Tyr/Asn194→Asp mutants of BHA. All constructs contained an N-terminal signal peptide, a C-terminal "foldon" sequence and a His-tag at the extreme C-terminus. The recombinant vaccinia viruses were generated in CV-1 cells and the all the constructs mainly expressed the unprocessed HA0. The CV-1 cells were maintained in DMEM medium supplemented with 10% FBS. For a typical preparation, 400 mL confluent CV-1 monolayer cells were infected at multiplicity of infection (MOI) of 1. After three days, the supernatant was collected and dialyzed. The soluble BHA was recovered from the supernatant by HisPur Cobalt Resin (Thermo Fisher Scientific Inc.) The beads bound with BHA were treated with neuraminidase (Sigma) to prevent the sialic acid-mediated aggregation in the following concentrating steps. The BHA was eluted with 500 mM Imidazole and was exchanged to buffer of 20 mM Tris-HCl (pH 7.2), 50 mM NaCl, then subjected to anion-exchange (mono-Q 4.6/100 PE, GE Healthcare) and gel-filtration chromatography (Superdex 200 10/300 GL, GE Healthcare). Only the peak according to the BHA-trimer was collected for the glycan assays and red blood cell binding assays. The protein concentration was qualified by Bradford protein assay. For the Asn194→Asp and Phe95→Tyr/Asn194→Asp mutants, the glycosylation site was

depleted in order to mimic the situation that the influenza B virus lost its glycosylation site at this position after egg adaptation. Correspondingly, the molecular weights of the HA0 monomer for these two mutants were about 3 kD smaller than that of wild type BHA as shown on the SDS-PAGE (data not shown).

2.2. Dose-dependent glycan assay

To characterize the receptor binding properties of BHA and its mutants, biotinylated α(2,3) (3'SLN, 3'SLN-LN and 3'SLN-LN-LN) and α(2,6) (6'SLN and 6'SLN-LN) glycans from the Consortium of Functional Glycomics (www.functionalglycomics.org) were used in the dose-dependent glycan assay. LN represents lactosamine (Galβ1-4GlcNAc), 3'SLN and 6'SLN represent Neu5Acα2-3 and Neu5Acα2-6 linked to LN, respectively. The different glycans were first diluted to 2.4 μM and loaded to the streptavidin-coated high binding capacity 384-well plates (Pierce), followed by incubating at 4 degree overnight. The excessive glycans were removed by washing with PBS for three times. The pre-complex of BHA, mouse anti-His antibody (0.2 mg/ml, Sigma) and anti-mouse-IgG antibody (2 mg/ml, Sigma) was prepared in a molar ratio of 4:2:1. The mixture was incubated on ice for 20 minutes, and the pre-complex was diluted to the expected concentration with 1% BSA in PBS. Each glycan-coated well was then loaded with 50 μl of the pre-complex of BHA and incubated at room temperature for 2 hours followed by wash with 0.05% Tween-20 in PBS to remove the unbound pre-complex. The Amplex Red Peroxidase Assay (Invitrogen) was used to measure the binding signal based on the HRP activity.

Negative controls, where no pre-complexes were loaded in those wells, were included for each glycan and the assays were performed in triplicate. To quantify the binding affinity, the assays were also performed at lower concentration range (0.05 to 1 μg/ml) for 3'SLN-LN and 6'SLN-LN. The Hill equation was used to represent the binding of the pre-complex of BHA to the coated glycan.

2.3. Red blood cell binding assay

Thirty micro-liter Ni-NTA resin (Thermo Fisher Scientific Inc.) was loaded with 2.5 μg BHA and incubated at room temperature for 2 hours. The beads were centrifuged down and the unbound BHA in supernatant was removed. The beads were then blocked with 3% BSA in PBS for 30 minutes. The supernatant was removed after spinning down the beads and the beads were washed with PBS extensively. The red blood cells (RBCs) were washed with Alsever's solution until the supernatant turns to be clear. The RBCs were stored as 50% in Alsever's solution after the final wash. The RBCs were then added to the BHA-coated beads in the final concentration of 0.5% and incubated at room temperature for 30 minutes. Finally, the beads were washed gently with PBS for appropriate imaging under microscope.

2.4. Influenza A virus infection inhibition assay in MDCK cells

For this assay, the BHA eluted from the Cobalt resin was used. The purity of BHA was about 85% to 90% as determined by the SDS-PAGE. The MDCK cells were seeded at appropriate concentration in 96-well plate one day before to achieve the

100% confluency when the assays were performed. The cell monolayer was washed with DPBS twice and complete DMEM (0.2% BSA, 25 mM HEPES in DMEM) once. The expected amount of BHA was loaded into different wells and complete DMEM was added to make all the wells contain same volume of supernatant. After the plate was incubated at room temperature for 1 hour, 5 HAU per 50 μl 1% human RBC of influenza A/Brisbane virus and TPCK-trypsin (final concentration was 2 μg/ml, Worthington) were added to wells and the plate was incubated at 37 degree for 1 hour (5% CO2). The supernatant was removed and the wells were washed twice with complete DMEM. Finally, each well was loaded with 100 μl influenza virus growth medium (complete DMEM with 2 μg/ml TPCK-trypsin). After 24 hours of infection, the supernatant was centrifuged (300 g, 15 minute) to pellet the cellular debris and used for the standard hemagglutination assay with 1% human RBC. All the assays were performed in triplicate.

2.5. Cell-based ELISA assay

In order to quantitatively detect the binding of receptors on the cell surface to BHA, cell-based Elisa assay was carried out with three cell lines including MDCK, BHK21 and Vero. For this assay, the BHA eluted from the Cobalt resin was used. The confluent cell monolayer in white 96-well plate (FALCON) was first washed with DPBS and expected amount of BHA diluted with 1% BSA in DPBS was added to different wells, complete DMEM was added to make all the wells have the same volume of solution. The plate was incubated at room temperature for 1 hour. The

supernatant was removed and each well was washed with DPBS for three times followed by being added 100 μl 3.7% formaldehyde diluted in DPBS for 10 minutes. The fixative was removed, and wells were washed with PBST (0.1% Triton X-100 in PBS) for three times. The wells were then blocked with 10% FBS for 1 hour at room temperature followed by incubation with primary antibody (mouse anti-His antibody, Sigma, 1:4000 dilution with 1% BSA in PBST) for 1 hour at room temperature. The wells were then washed three times with PBST. The wells were finally incubated with secondary antibody (HRP-conjugated anti-mouse-IgG, Sigma, dilution 1:40000) for 1 hour at room temperature. After washing with PBST and PBS, the binding signal was measured with ECL reagent (Amersham). All the assays were performed in triplicate.

3. Results

3.1. Results of glycan assay

Using recombinant BHA expressed in mammalian cells by vaccinia virus system (13), we first performed dose-dependent glycan binding assay using three α(2,3)-linked avian-like receptors and two α(2,6)-linked human-like receptors. It was apparent that the wild-type BHA preferentially binds to the long α(2,6)-linked human-like receptor (6'SLN-LN) (Fig. 1a). The mutation Phe95→Tyr simultaneously enhanced the binding for both avian-like (3'SLN-LN-LN) and human-like (6'SLN-LN) receptors in the long format (Fig.1b). The calculated apparent binding constants for the wild-type and Phe95→Tyr BHA were 13.4 mM and 2.44 μM for the 3'SLN-LN receptor, and 0.13 mM and 10.5 pM for the 6'SLN-LN receptor, respectively (Table 1). Not surprisingly, the mutation Asn194→Asp, which causes the loss of a glycosylation site at HA1 194~196, as frequently found in egg-adapted variants (6-12), results in the preferential binding to α(2,3)-linked avian-like receptors, with an apparent binding constant of 2.9 pM for 3'SLN-LN, in contrast to the 0.209 mM binding constant for α(2,6)-linked 6'SLN-LN (Fig.1c, Table 1). As seen for the single Phe95→Tyr mutant, the double mutant Phe95→Tyr/Asn194→Asp improves the binding for both α(2,3)-linked and α(2,6)-linked receptors (Fig.1d), with apparent binding constants of 0.45 pM and 1.90 pM for 3'SLN-LN and 6'SLN-LN, respectively (Table 1). Thus, regardless of whether or not there is a glycosylation at HA1 194~196, the mutation Phe95→Tyr enhances the binding of BHA for both avian and human-like receptors in an indiscriminative manner.

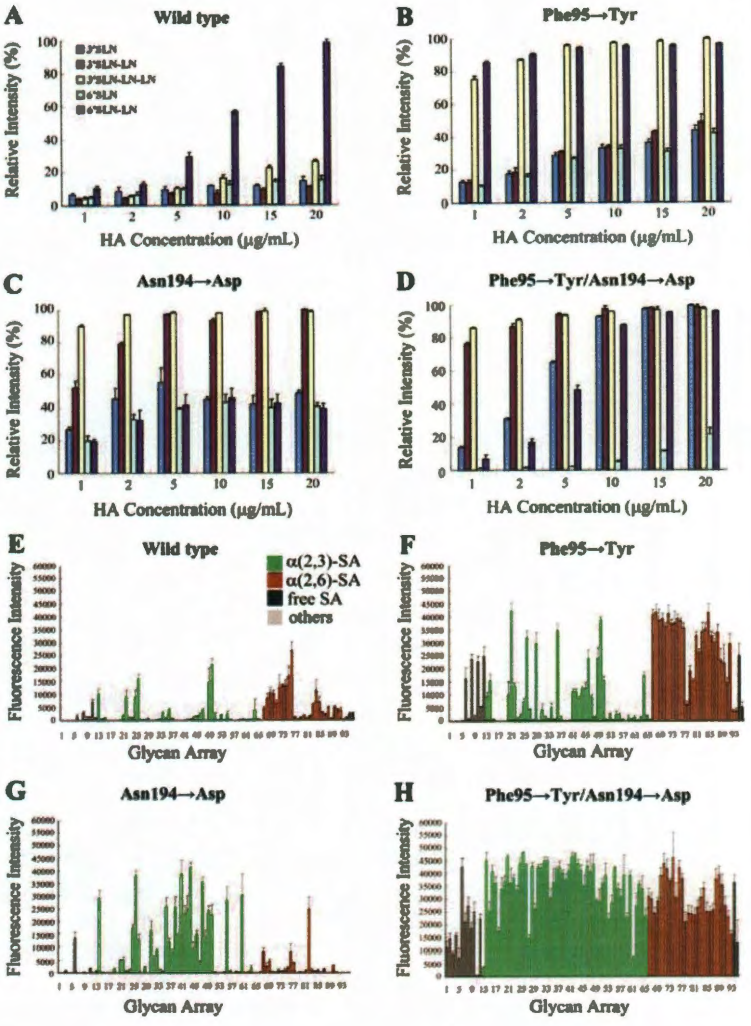

Fig.1. The mutation Phe95→Tyr promotes the binding of BHA to synthetic glycans in dose-dependent glycan assay (A-D) and glycan microarray analysis (E-H). The five glycans used in the dose-dependent assay are 3'SLN (■), 3'SLN-LN (■), 3'SLN-LN-LN (■), 6'SLN (■) and 6'SLN-LN (■). See *Materials and Methods* for the full name of each glycan. The binding signals (A-D) are expressed as the percentage of the maximum fluorescence intensity. Wild type BHA shows significant binding only to long α(2,6) (6'SLN-LN), while its Phe95→Tyr mutant binds to long α(2,3) (3'SLN-LN-LN) and long α(2,6) (6'SLN-LN) almost identically (Compare B with A). With the loss of glycosylation site at 194, the Asn194→Asp mutant shows higher binding to long α(2,3) (3'SLN-LN and 3'SLN-LN-LN) (Compare C with A), while the binding capacity to long α(2,6) (6'SLN-LN) of the Phe95→Tyr/Asn194→Asp mutant increases (Compare D with C). Only those glycans with absolute fluorescence intensities above 5000 are shown for the glycan microarray assay (E-H). The glycans are categorized into α(2,3)-linked sialosides (■), α(2,6)-linked sialosides (■), free sialic acid (■) and others (■). The full names of these glycans are listed in Table S1. Wild type BHA binds to limited number of glycans, while its Phe95→Tyr mutant can bind to a few more α(2,3) and α(2,6) (Compare F with E). The loss of glycosylation site at 194 causes the Asn194→Asp mutant to bind more α(2,3) but less α(2,6) (Compare G with E). The Phe95→Tyr/Asn194→Asp mutant binds to the most number of α(2,3) and α(2,6) (H).

To analyze further the glycan binding properties of Phe95→Tyr mutants, we performed glycan microarray assay using 465 different glycans. Shown in Fig.1e~h are the binding profiles for 94 glycans that yielded significant binding signals in the glycan microarray assay. All these 94 glycans had terminal sialic acid groups. Consistent with the results of dose-dependent glycan binding assays, the wild-type BHA preferentially binds to α(2,6)-linked receptors (Fig.1e, red colors), while Asn194→Asp preferentially binds to α(2,3)-linked receptors (Fig. 1g, green colors). The mutants Phe95→Tyr and Phe95→Tyr/Asn194→Asp have much stronger binding for both α(2,6)-linked and α(2,3)-linked receptors (Fig.1f, 1h). It is worth noting that BHA proteins with a glycosylation at HA1 194~196, the wild type and Phe95→Tyr mutant, had an overall weaker binding for glycans than their counterparts without a glycosylation (Fig.1a~h, Table 1), agreeing with an earlier observation.

Table 1 The relative binding affinities of BHA to α(2,3) and α(2,6) receptors

BHA	3'SLN-LN			6'SLN-LN		
	n	Kd'	R^2	n	Kd'	R^2
Wild type	0.426	13.4 mM	0.9816	0.604	0.134 mM	0.9714
Phe95→Tyr	0.868	2.44 μM	0.9969	1.26	10.5 pM	0.9970
Asn194→Asp	1.49	2.90 pM	0.9982	0.728	0.209 mM	0.9877
Phe95→Tyr/Asn194→Asp	1.45	0.45 pM	0.9800	1.65	1.90 pM	0.9710

The apparent binding constant Kd', cooperativity factor n and the R-square R^2 were obtained by fitting the data to the linear Hill equation in order to quantitatively determine the relative binding affinities of BHAs, and their absolute values should be compared only in this context.

One interesting observation from the glycan microarray results was that the Phe95→Tyr mutant displays substantial enhanced binding for two free sialic acids of different lengths (shown in black colors in Fig.1e~h), suggesting that the gain in

binding to receptors by Phe95→Tyr is probably the result of a much tighter binding between the extra hydroxyl group of the introduced Tyr95 with the terminal sialic acid on the receptors, which would also help explain its indiscriminative binding for both human and avian-like receptors. To explore this explanation further, we made a Phe95→Tyr mutation on our previously published structures of BHA-receptor complexes in silico (Fig.2) (14). By using the second most popular side chain conformation and a small adjustment in torsion angles, Tyr95 in BHA was easily placed at a position similar to Tyr98 in the structure of AHA H3 subtype without any potential structural clash (Fig.2). At this position and without any structural adjustment of the terminal sialic acid, the newly added hydroxyl group of Tyr95 forms two hydrogen bonds with the terminal sialic acid and one additional bond with His191 (Fig.2 in red dashed lines). Thus, these extra hydrogen bonds introduced by the Tyr95 residue are most likely the molecular basis for the improved binding affinity of Phe95→Tyr with receptors.

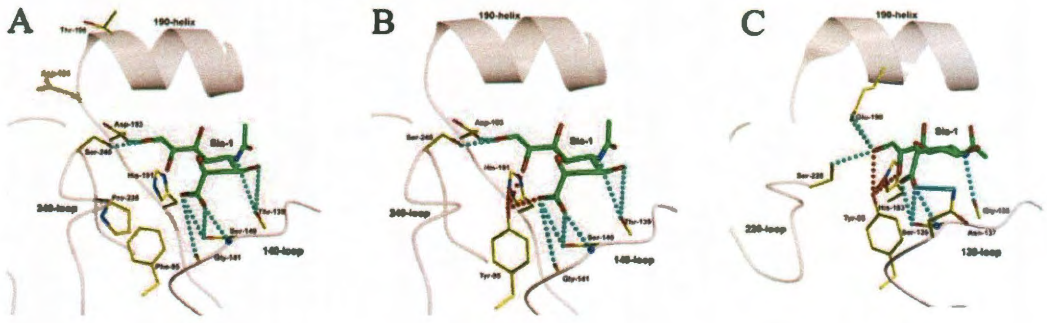

Fig.2. Sialic acid binding with BHA (A), model structure of Phe95→Tyr mutant (B) and AHA (C). In the model structure of Phe95→Tyr mutant, the hydroxyl group of Tyr95 could form two more hydrogen bonds with sialic acid moiety and one more hydrogen bond with His191 as compared with those in wild type BHA crystal structure (A). These three extra hydrogen bonds exist in the crystal structure of AHA (C).

3.2. Results of red blood cells binding assay

Red blood cells from different species are known to contain varying amounts of avian and/or human-like receptors. Thus, if the enhanced binding affinity of Phe95→Tyr for synthetic receptors holds true, we would expect that this mutation allows better binding for natural sialic acid receptors on many different red blood cell types. Toward this end, we tested red blood cells from eight species (Fig.3). All four proteins were found to bind well to human red blood cells. Compared with their corresponding wild-type proteins, both Phe95→Tyr and Phe95→Tyr/Asn194→Asp mutants bound significantly better for red blood cells from chicken, turkey, and guinea pig, and somewhat better for those from rabbit and bovine. For the red blood cells from swine, the mutant Phe95→Tyr bound significantly better while Phe95→Tyr/Asn194→Asp only bound slightly better than their corresponding wild-type proteins (Fig.3). Therefore, the higher binding affinity of the Phe95→Tyr and Phe95→Tyr/Asn194→Asp mutants for synthetic receptors can be readily translated into a much stronger binding to natural sialic acid receptors on red blood cells.

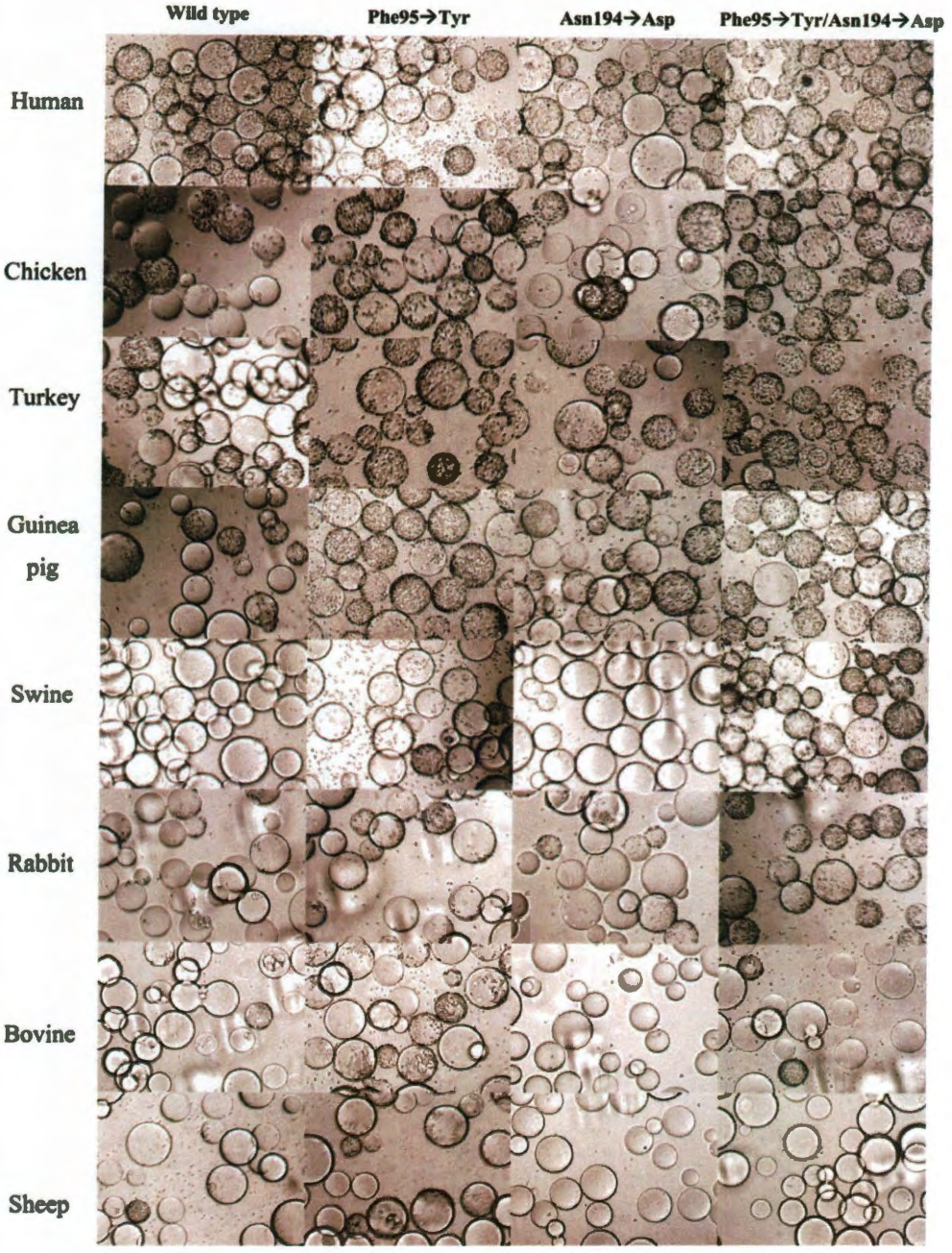

Fig.3. Red blood cell (RBC) binding assay. The species are listed on the left. Wild type BHA and its mutants bind equally well to human RBCs. The Phe95→Tyr and Phe95→Tyr/Asn194→Asp mutants show better binding toward chicken, turkey, guinea pig, swine and rabbit RBCs than wild type BHA and the Asn194→Asp mutant, respectively. There are no big binding differences with bovine and sheep RBCs for wild type BHA and its mutants.

3.3. Results of influenza A virus infection inhibition assay in MDCK cells

Since binding to cell-surface receptors is a prerequisite for infecting host cells by influenza virus, we asked whether the much higher binding affinity of the Phe95→Tyr and Phe95→Tyr/Asn194→Asp mutants would allow them to compete more effectively against infection caused by influenza virus. As a most stringent test, we used influenza A/H3N2 virus (A/Brisbane/2007) that generally has much higher binding affinity for cell-surface receptors than influenza B virus. The test also included a "blank" where no influenza A virus was added and a "positive control" where only influenza A virus was added (Fig.4). Using five hemagglutination units (HAU) of influenza A virus as input to infect Madin Darby Canine Kidney (MDCK) cells, at 48 hour post-infection, the "positive control" had a virus titer of about 32 HAU per 50 μL 1% human red blood cells. With 400 μg recombinant proteins, the Phe95→Tyr mutant displayed four-fold stronger inhibition of influenza A virus than its wild type, which had about two-fold inhibition over the "positive control". Most strikingly, the mutant Phe95→Tyr/Asn194→Asp completely blocked the infection of influenza A virus, with as low as 200 μg (Fig.4) and 100 μg (data not shown) recombinant proteins. In marked contrast, with 200 μg recombinant protein, the Asn194→Asp yielded a virus titer of 32 HAU per 50 μL 1% human red blood cells (Fig.4). Although MDCK cells have both α(2,6)-linked and α(2,3)-linked sialic acid receptors, it was speculated that the α(2,3)-linked sialic acid receptors are in a much low abundance than α(2,6)-linked sialic acid receptors (15). This may explain the roughly two-fold inhibition displayed by the wild-type BHA that preferentially binds to α(2,6)-linked sialic acid receptors, in contrast to the null inhibition exhibited by the

Asn194→Asp mutant (Fig.4). Therefore, the higher binding affinity of the Phe95→Tyr and Phe95→Tyr/Asn194→Asp mutants for synthetic and natural sialic acid receptors did allow a much stronger competition against influenza virus infection.

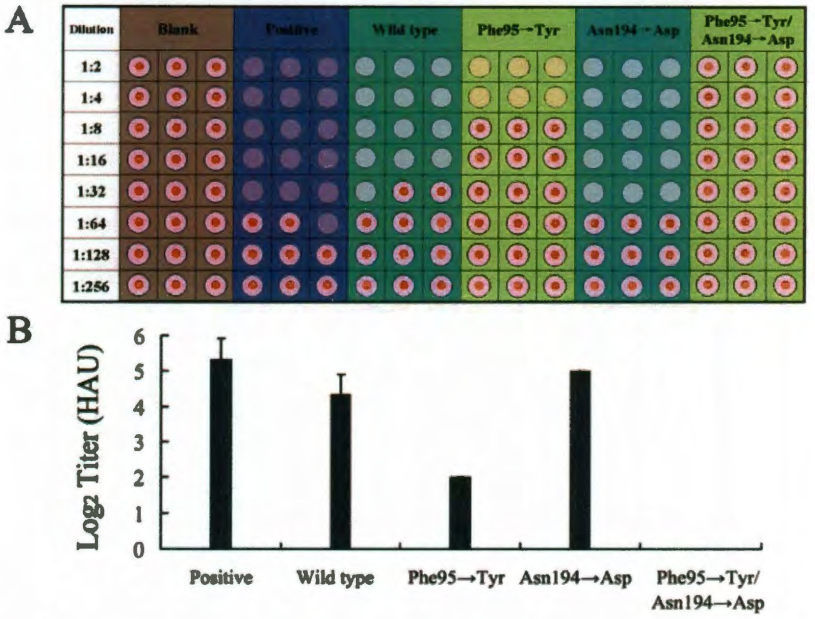

Fig.4. The mutation Phe95→Tyr efficiently inhibits the binding of influenza A virus to MDCK cells. The "Blank" sample is the negative control where the MDCK cells grow under normal condition in the binding inhibition assay. The "Positive" sample is the positive control where the MDCK cells are infected with 5 HAU influenza A virus. The "Wild type" and "Phe95→Tyr" samples represent those wells where the MDCK cells are first binding with 400 μg wild type BHA and Phe95→Tyr mutant before the infection of 5 HAU influenza A virus, respectively. The "Asn194→Asp" and "Phe95→Tyr/Asn194→Asp" samples are for those wells where the MDCK cells are first binding with 200 μg Asn194→Asp and Phe95→Tyr/Asn194→Asp mutants before the infection of 5 HAU influenza A virus. The supernatants after 24-hour incubation are used for the hemagglutination assay (upper figure). If there are enough viruses at certain dilution, the lattice will form due to the interactions (agglutination) between RBC and hemagglutinin on the surface of influenza A viruses (shown as pink circle); otherwise the RBC will settle to the bottom of the well to form a red button (shown as a small red circle in a big pink circle). The titer is read as the endpoint of agglutination (lower figure). The titers are much smaller if Phe95 is mutated to Tyr95 as compared Phe95→Tyr with wild type or Phe95→Tyr/Asn194→Asp with Asn194→Asp, meaning that the virus could not grow well if Phe95→Tyr or Phe95→Tyr/Asn194→Asp is present. This indicates that the Phe95→Tyr and Phe95→Tyr/Asn194→Asp mutants could compete against the hemagglutinins on the influenza A virus in the binding to the receptors on the MDCK cells, thus inhibit the entrance of influenza A virus.

3.4. Results of cell-based ELISA

Based on the results of influenza virus infection inhibition assay presented above, we asked whether there is a correlation between receptor-binding affinity of BHA and the host range of influenza B virus. In other words, would the enhanced receptor-binding affinity of the Phe95→Tyr and Phe95→Tyr/Asn194→Asp mutants allow BHA to bind a number of host cells significantly better? To explore this, we developed a whole-cell-based ELISA assay that quantitatively measures the amount of BHA proteins immobilized on cell culture monolayers due to their association with cell-surface receptors (see Methods for details). Three established cell lines were used: MDCK, Baby hamster kidney (BHK21), and African green monkey kidney (Vero). Although MDCK is a well-established cell line for influenza A and B viruses, mixed successes were obtained for BHK21 and Vero in supporting influenza B virus growth (16). The wild-type BHA generally had a very weak binding to all three cell lines, with the weakest for BHK21 cell line (Fig.5). The mutation Phe95→Tyr substantially improved the binding to all three cell lines, while the most significant improvement was seen for the Vero cell line (Fig.5). The Asn194→Asp mutant had a similar binding affinity as the Phe95→Tyr on the wild-type background, but the Phe95→Tyr/Asn194→Asp enhanced the binding affinity by around 1000-fold over those of Asn194→Asp (Fig.5). Taken together, the mutation Phe95→Tyr allowed for a much tighter binding to all the three cell lines tested here, thus satisfying the first prerequisite for the ability of influenza B virus to bind and infect a wide range of hosts.

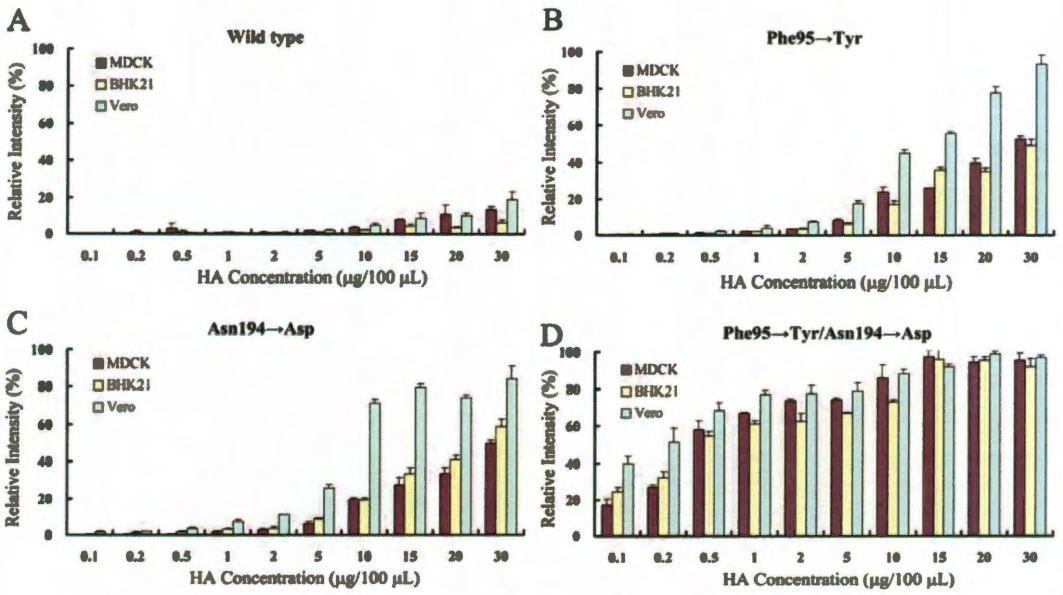

Fig.5. The mutation Phe95→Tyr enhances the binding of BHA to the surface of cultured cells. The binding signals are expressed as the percentage of the maximum chemiluminescence intensity for MDCK (A), BHK21 (B) and Vero (C) in cell-based ELISA. In all three cell lines tested, wild-type BHA has the weakest binding, while the Phe95→Tyr/Asn194→Asp mutant shows the strongest binding.

4. Concluding Discussion

It is well established that naturally occurring influenza B virus has much lower binding affinity for synthetic glycans and natural receptors on cell surface, and it also has a very limited host range, infecting mostly humans. It remains an open question whether there exists a correlation between the limited host range and the lower receptor binding affinity. We use dose-dependent glycan assay and glycan microarray assay to show that the mutant Phe95→Tyr could increase the binding of BHA with synthetic glycans, and red blood cell binding assay to show that this mutation increases the binding of BHA with natural receptors. The higher binding affinity of the Phe95→Tyr mutant for synthetic and natural sialic acid receptors did allow a much stronger competition against influenza virus infection as shown in the influenza A virus infection binding assay. Further, the Phe95→Tyr mutant could bind with different host cell lines with high affinities. Together, these results suggest that the residue at position 95 should be the key for revealing the mechanism of limited host range and pathogenicity of influenza B virus.

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